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ORIGINAL ARTICLE

Factors regulating mandibular condylar growth

A. B. M. Rabie, MSc, CertOrtho, PhD, FCDSHK, FHKAM,[a] and
U. Hägg, DDS, OdontDr, Cert Comp Orth, FHKAM, FDSRCS[b]
Hong Kong SAR, China

Factors regulating condylar growth have not been identified before. This study was designed to identify a series of these factors, such as Sox 9 transcription factor and vascular endothelial growth factor (VEGF), and also to correlate the amount of type X collagen expressed during natural growth to the amount of bone newly formed. We used 115 Sprague-Dawley rats, 35 days old, in this study. The expression of these factors was identified on protein level by using immunostaining. Type X collagen was identified on mRNA and protein levels. Sox 9 was expressed by cells in the proliferative layer and by chondrocytes. Type X was expressed only by hypertrophic chondrocytes, and its expression precedes the onset of endochondral ossification. VEGF is expressed by hypertrophic chondrocytes, and its maximum level of expression precedes the maximum level of bone formation. Condylar growth involves a sequence of transitory stages uniquely defined by molecules that are intrinsically synthesized by cells in the condyles. (Am J Orthod Dentofacial Orthop 2002;122:401-9)

Much research has been reported concerning the development of the mandible, specifically the mandibular condyles.[1-6] The main studies of the past 30 years have dealt with natural growth of the mandibular condyles and growth changes during the use of different orthopedic appliances on experimental animals.[7-12] This work included studies that identified cellular influences on condylar growth[7] and the mode of action of growth hormones on different varieties of cartilage,[8] and the leading autoradiographic studies of Petrovic et al[9-11] on identifying cell proliferation and DNA synthesis in many treatment modalities. The work dealt with condylar growth on a cellular level, which resulted in several questions that can only be answered today on a molecular level.

Petrovic and Stutzmann[9] hypothesized that "There must be a negative feedback-signal originating from the proximal part of the chondroblastic zone and exerting a restraining effect on the prechondroblastic multiplication rate." In other words, they suggested that condylar growth is regulated in part by factors that are intrinsically expressed. As of today, most of these regulatory factors have not been identified in the condyles, unlike the long bones in which many regulatory factors have already been identified.

Several differentiation factors, growth factors, and angiogenic mediators have been found to play important roles during endochondral ossification of long bones.[13-21] Similarly, condylar growth must be regulated by a host of orchestrated influences of various growth factors and other regulatory factors that are endogenously expressed in the condyles. The identification of these factors in the condyles could help to answer several key questions concerning condylar growth: What regulates cellular differentiation in the condyles during growth? What triggers the onset of cartilage ossification? How does bone replace cartilage in the condyles? What is the nature of the matrix of endochondral ossification in the condyle during growth?

In a series of experiments to identify the master transcription factors that control the differentiation of mesenchymal cells into chondrocytes during development of long bones, it was demonstrated that the transcription factor Sox 9 is required for chondrocyte differentiation and for expression of a series of cartilage-specific marker genes including types II, X, and XI collagens.[22,23] The expression of Sox 9 has not been identified in the growing mandibular condyles before. To place Sox 9 function during differentiation of mesenchymal cells in growing condyles, its expression by cells within the condyles should be identified and correlated with the cellular events occurring during endochondral ossification of the condyles.

Once mesenchymal cells differentiate into chondro-

From the Department of Orthodontics, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, Hong Kong SAR.
[a]Associate professor.
[b]Chair and professor.
This work was supported by CRCG Grants 10201953 15633 and 10201960 22311.
Reprint requests to: Dr A. M. Rabie, Orthodontics, Faculty of Dentistry, The University of Hong Kong, Prince Philip Dental Hospital, 34 Hospital Rd, Hong Kong SAR, China; e-mail, rabie@hkusua.hku.hk.
Submitted, September 2001; revised and accepted, December 2001.

0889-5406/2002/$35.00 + 0 8/1/125713
doi:10.1067/mod.2002.125713

cytes in the condyles, they mature, form cartilage, and express type II collagen, a major component of the cartilaginous matrix of the condyles.[14]

Chondrocytes later undergo hypertrophy and express type X collagen as do the hypertrophic chondrocytes in the growth plate cartilage.[15,16] Type X collagen has been used as a marker for endochondral ossification of the long bones and the mandibular condyles.[16,17] Its expression precedes the onset of endochondral ossification, and it forms the matrix of the hypertrophic cartilage destined for ossification. Therefore, in this study, we correlated the temporal expression of type X collagen on molecular and protein levels in the growing condyles to the amount of bone expressed during condylar growth.

Osteogenesis (the formation of new bone) and angiogenesis (the invasion of new blood vessels) are closely correlated.[18] Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is actively responsible for hypertrophic cartilage neovascularization in long bones by inducing endothelial cell migration and proliferation.[19] Therefore, angiogenesis is essential for the replacement of cartilage by bone during growth of the long bones.[20,21] Horner et al[20] reported that the presence of numerous small blood vessels and vascular structures in the subchondral region where VEGF expression was maximal indicated that the VEGF produced by hypertrophic chondrocytes might be a key to the regulation of vascular invasion of the growth plate in long bones. To obtain a better understanding of the mechanisms regulating vascular invasion at sites of endochondral ossification in the condyles, we investigated the expression of the endothelial cell-specific mitogen, VEGF, by cells of the condyles in growing rats.

The purposes of this study were (1) to identify the cells expressing Sox 9 transcription factor in the condyles of growing rats and to correlate its expression to cellular events occurring during condylar growth, (2) to correlate the temporal expression of type X collagen in the condyles of growing rats to the amount of bone expressed during condylar growth, (3) to identify the cells expressing VEGF in the condyles of growing rats and to correlate its expression to endochondral ossification of the condyles, and (4) to quantify the amount of Sox 9, type X, and VEGF expressed during natural growth, hoping to develop a baseline against which changes in the condyles during different modalities of treatment can be compared in later experiments.

MATERIAL AND METHODS

We used 115 female Sprague-Dawley rats, 5 weeks of age, in this study. They were divided randomly into 4 groups, 25 animals per group, except for the Sox 9 group, which contained 40 rats. They were fed a soft diet and were later killed at 38, 42, 49, 59, and 65 days of age.

In situ hybridization with paraffin sections was performed with modification as described by Wilkinson[24] and Kwan et al[25] to determine the spatial expression of type X collagen mRNA transcripts in situ. Sense and antisense [α-^{35}S]-UTP riboprobes were generated by in vitro transcription from linearized DNA template using RNA Polymerase T3, T7, and SP6 in transcription buffer (40 mmol/L Tris-HCl, pH 8.5, 6 mmol/L $MgCl_2$, 2 mmol/L spermidine) with 10 mmol/L DTT, 250 μmol/L ATP, CTP, GTP, 50 units of Rnasin, 100 μCi [α-^{35}S]-UTP in a total volume of 20 μL incubated at 37°C for 1 hour. The DNA template was removed by adding 40 units of DNase I (RNase free) into the reaction mixture at 37°C for 1 hour. Unincorporated [α-^{35}S]-UTP was removed by column chromatography in a Sephadex G50 column. The column was then eluted with aliquots of 200 μL of column buffer. A total of 8 aliquots of 200 μL eluant were collected into individual eppendorf tubes. The radioactivity of each tube was determined by liquid scintillation counting. The RNA probe was precipitated out from the tube with the highest radioactivity by adding 0.5 volume of 6 mol/L NH_4Ac, pH 5.3, and 2 volume of absolute ethanol. The riboprobe was collected by centrifugation (10,000 *g*, 30 min at 4°C), washed in 80% ethanol, and dried. The labeled riboprobe was resuspended in 0.1 mol/L DTT to give a radioactivity of 2×10^6 cpm/μL followed by adding 9 volume of hybridization buffer and stored at −20°C.

The tissue sections were dewaxed and then fixed briefly in 4% (w/v in PBS) paraformaldehyde. The sections were treated with PK (20 μg/mL in TE) for 5 minutes to facilitate the penetration of riboprobe into the sections, washed with PBS for 5 minutes, and fixed again with 4% (w/v in PBS) paraformaldehyde for 20 minutes; then they were ready for hybridization with riboprobe.

The sense and antisense ^{35}S-labeled riboprobe dissolved in hybridization buffer were heated at 80°C for 3 minutes, and small amounts of each were applied onto the pretreated sections on the glass slides, and a clean cover slip was placed on the sections to spread the riboprobe uniformly. The hybridization was carried out in a slide box put into a sealed jar with 50% (v/v) deionized formamide and 5× SSC at 50°C for 18 hours. The slides treated with sense riboprobe served as negative controls.

The hybridization signal was detected by autoradiography by dipping the slide in liquid emulsion K5

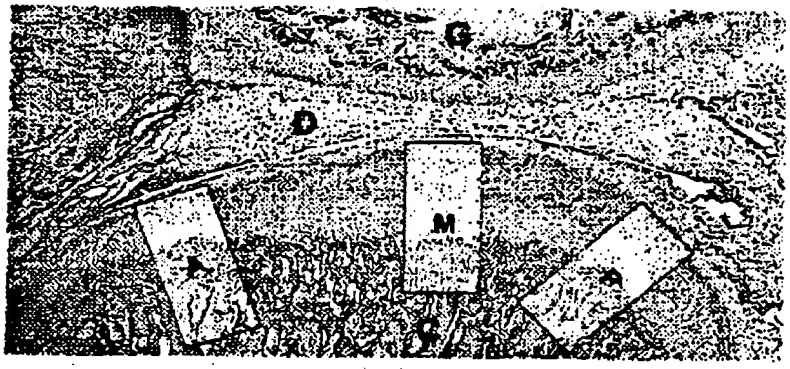

Fig 1. Magnification of anterior (*A*), middle (*M*), posterior (*P*) regions of condule (*C*), glenoid fossa (*G*), and articular disk (*D*) at 36 days of natural growth.

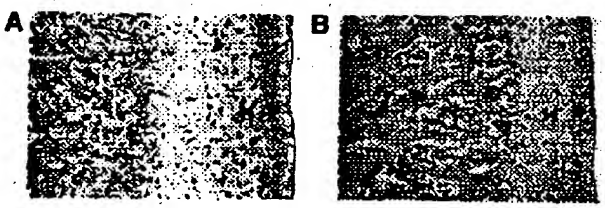

Fig 2. Expression of Sox 9 gene in growing rat condyles (natural growth). **A**, Day 40; **B**, day 44. Hypertrophic (*H*) and proliferative (*P*) layers.

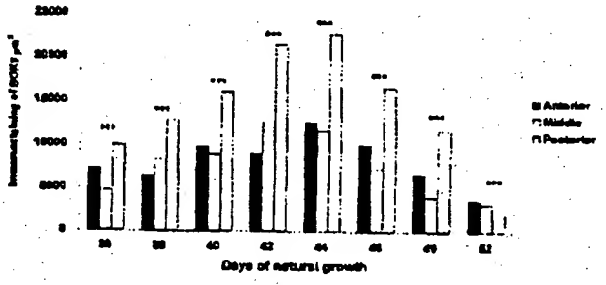

Fig 3. Temporal pattern of expression of Sox 9 in proliferative zone of anterior, middle, and posterior regions of condyle from day 36 to day 52 during natural growth.

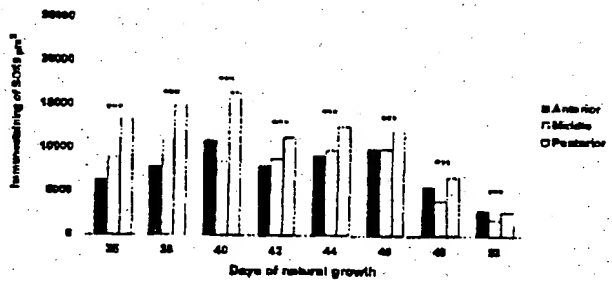

Fig 4. Temporal expression of Sox 9 in hypertrophic zone of anterior, middle, and posterior regions of condyle from day 36 to day 52 during natural growth.

(Ilford Imaging, Paramus, NJ). The emulsion was allowed to dry; the emulsion-coated slides were put into a slide box, sealed, and exposed for 7 days at 4°C before developing. After exposure, the slides were equilibrated to room temperature, and the sections were developed in Ilford Phenisol developer for 2 minutes followed by stopping with 0.5% (v/v) acetic acid for 1 minute and fixing in 30% (w/v) sodium thiosulphate for 6 minutes. The developed sections were stained for histology in hematoxylin and eosin. Sections w re photographed with Kodak Ektachrome ASA 64 film on a microscope under bright-field illumination with a blue filter.

Immunohistochemistry

The primary antibody for type X collagen was provided by Dr G. J. Rucklidge of Rowett Research Institute, Aberdeen, Scotland. The antisera raised against type X collagen was found not to cross-react with other collagen types when tested either by ELISA r immunoblotting as reported by Rucklidge.[25]

Fig 5. Expression of type X collagen by hypertrophic chondrocytes in growing rat's condyles-natural growth (courtesey of *Quintessence*[16]).

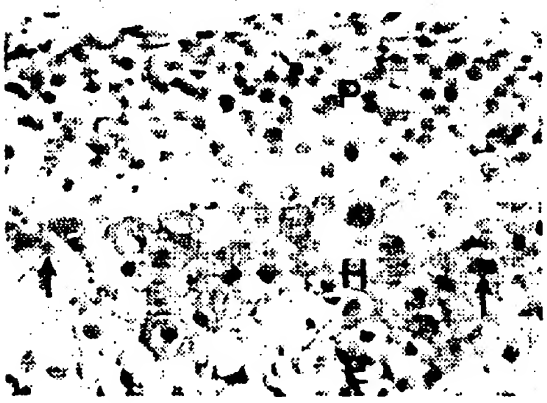

Fig 6. Expression of type X collagen by hypertrophic chondrocytes in growing rat's condyles during natural growth. Immunostaining (*arrows*) localized in hypertrophic (*H*) layer, and not expressed in proliferative (*P*) layer or erosion (*E*) zone. (courtesey of *Quintessence*[16]).

The secondary antibody used was anti-rabbit IgG-peroxidase conjugate preadsorbed with normal fetal bovine serum (Sigma Code No. A-4914). Immunoperoxidase staining was performed as described by Ruck lidge[26] with a slight modificati n.

The primary antibody for Sox 9 was provided by Dr Vincent Harley, Prince Henry's Institute of Medical Research, Monash, Australia. After the sections were dewaxed and rehydrated, they were treated in 3% H_2O_2 for 10 minutes followed by protease digestion (Proteinase K, Sigma P-6556, 10 μg/mL). The sections were then incubated with normal goat serum (Sigma G9023, 1:9 diluted with 1xTBS), polyclonal rabbit anti-human Sox 9 and biotin-conjugated goat-anti-rabbit IgG (Sigma B-8895), 1:200 preadsorbed with normal goat serum successively each for 1 hour at 37°C. They were washed with PBS between each step. The sections were developed in DAB (3,3-diaminobenzidine, Sigma D-5637) for 1 minute and counter-stained with Mayer hematoxylin for 3 minutes.

Immunolocalization for the VEGF was performed with goat polyclonal IgG preparations (Sigma V1253). Before adding the primary antibody, the antigenic sites were exposed by digestion with trypsin for 20 minutes at 37°C in 3% BSA (Sigma). Excess buffer was rinsed off, and the primary antibody was applied at a concentration of 2 μg/mL (1:50) and incubated overnight at 4°C in a humidified chamber. Free antibodies were removed by washing with PBS, and secondary biotin-conjugated rabbit-anti-goat (Dako E0466) antiserum was applied. After washing, the sections were incubated at 37°C for 30 minutes in ABC reagent (Strept ABComplex/HRP, Dako K0377) and then developed in 0.05% DAB (3,3-diaminobenzidine tetrahydrochloride, Sigma D5637) for 5 minutes. The slides were then counterstained with Mayer hematoxylin, cleaned, and mounted with coverslips.

Quantitative evaluation

The expression of Sox 9, VEGF, and type X collagen, and the amount of bone newly formed during normal growth, were quantified by measuring the area of signals and the new bone with a computer-assisted image analyzing system (Leica Q550IW, Leica Microsystems Imaging Solutions Ltd, Clifton R, Cambridge, United Kingdom) with software (Leica Qwin Pro, Version 2.2), following the method reported by Rabie et al.[27]

RESULTS

Sox 9 transcription factor was expressed by cells in the proliferative and the hypertrophic zones as shown in Figures 1 and 2. Cells in the proliferative and hypertrophic zones in the posterior region of the condyles expressed more Sox 9 than in the anterior and middle

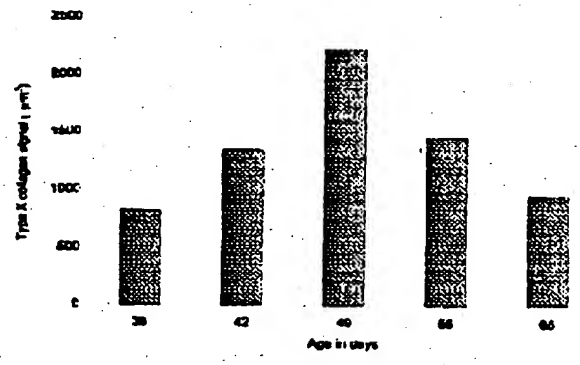

Fig 7. Temporal pattern of type X collagen mRNA expression in condylar cartilage during days 38 to 65 of natural growth (in situ hybridization).

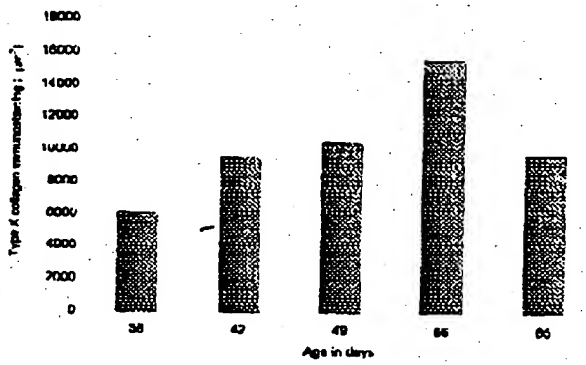

Fig 8. Temporal pattern of type X collagen expression in condylar cartilage during days 38 to 65 of natural growth.

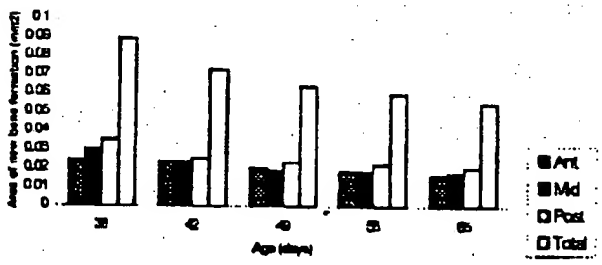

Fig 9. Bone formation in condyles during days 38 to 65 of natural growth (PAS staining).

zones of the condyle (Figs 3 and 4). The highest levels of expression of Sox 9 were reached on day 44 of growth in the proliferative layer and on day 40 in the hypertrophic layer (Figs 2-4).

Results of in situ hybridization and immunostaining

Fig 10. Expression of VEGF by hypertrophic chondrocytes in rat condyles during natural growth.

Fig 11. Localization of VEGF in cells of hypertrophic zone.

showed that type X collagen is secreted only by hypertrophic chondrocytes (Figs 5 and 6). The staining is localized in the hypertrophic layer (*H*), and no staining was identified in the proliferative layer (*P*) or the resting layer (*R*). The temporal pattern of expression of type X collagen mRNA showed that the highest level of expression was reached on day 49 (Fig 7), whereas that of type X collagen molecules was reached by day 56 (Fig 8). The type X collagen expression continued to precede the bone formation as shown in Figure 9.

VEGF was expressed by cells in the upper hypertrophic layer. (Figs 10 and 11). The posterior region maintained a significantly higher level of VEGF than any of the other 2 areas (Figs 12 and 13). The highest level was reached on day 38 followed by a gradual decrease. When these results were correlated to the temporal pattern of new bone formation, it was apparent that the highest amount of blood supply had to be

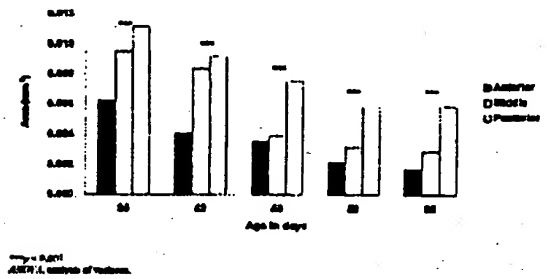

Fig 12. VEGF expression during natural growth.

Fig 13. Total amount of VEGF expression during natural growth.

reached before the greatest amount of new bone formed in the condyle during natural growth.

DISCUSSION

This study identified some cellular and molecular events that are responsible for key processes governing condylar growth. Condylar growth follows a sequence of transitory stages that are uniquely defined by molecules synthesized by undifferentiated mesenchymal cells and by differentiating chondrocytes (Figs 1-14).

In the developing rat condyles, mesenchymal cells, present in the proliferative layer, express Sox 9 transcription factor (Fig 1). Sox 9 is expressed in all cartilage primordial and cartilages, coincident with the expression of type II collagen, the major collagenous framework of cartilage. Bi et al[28] demonstrated that, in mouse chimeras, Sox 9–deficient mesenchymal cells are excluded from all cartilage but are present as mesenchymal cells that do not express type II collagen, the chondryocyte-specific marker. This clearly points to its essential role in the differentiation of mesenchymal cells into chondrocytes. Therefore, the expression of Sox 9 factor by the cells in the proliferative layer points to a role in regulating the differentiation of mesenchymal cells into chondrocytes in the condyles of growing rats. Sox 9 transcription factor was also expressed by the chondrocytes in the growing rats' condyles (Fig 2). Bi et al[28] reported that Sox 9 is required for cartilage formation. In humans, Sox 9 haploinsufficiency results in campomelic dysplasia, a severe skeletal malformation syndrome,[29] indicating a critical role for Sox 9 in skeletogenesis. Therefore, its expression by condylar chondroctyes points to a similar role in regulating the cartilage formation in the growing condyle. Furthermore, Sox 9 has been found to be a potent activator of type II collagen expression by chondrocytes: this is an essential component of the differentiation program of these cells.[30] Type II collagen has been localized in the mandibular condyles during growth[31] and in different mandibular postures.[32] It is important to identify the pattern of Sox 9 because it regulates the condylar cartilage formation through regulating the expression of its framework, the type II collagen. In our study, Sox 9 was found to be expressed in 36-day-old rats and continued to be expressed throughout the examined period until day 52 (Figs 3 and 4). A quantitative analysis with an image analyzer identified the amount of Sox 9 expressed during natural growth to create a baseline against which changes in the condyles during different modalities of treatment can be compared in future experiments.

After cartilage matrix formation, chondrocytes undergo hypertrophy; this marks the onset of endochondral ossification. In the condyles, hypertrophic chondrocytes engage in the synthesis of type X collagen.[16] Recently, in the Japanese literature, we reported that type X collagen was expressed only by the hypertrophic chondrocytes in the growing rat's condyles (Figs 5 and 6).[16] In the present study, we correlated the temporal expression of type X collagen to endochondral ossification in the condyles of growing rats (Figs 7-9). A close correlation existed between the expression of type X collagen mRNA, protein, and bone formation. The maximum level of type X collagen mRNA was reached on day 49, followed by the maximum level of type X collagen on day 56 (Fig 9). Naturally, the type X mRNA maximum level must precede the protein levels to allow the time required for the protein synthesis and secretion to the extracellular matrix. The expression of the hypertrophic cartilage matrix, comprised mainly of type X collagen, is closely related to the endochondral bone formation naturally occurring during condylar growth. These results support the findings of other reports showing that the expression of type X collagen preceded the onset of endochondral ossification in long bones and in fracture repair.[33,34] It was proposed that type X collagen forms the collagenous framework of the hypertrophic cartilage destined for endochondral ossification because it is easily resorbed when compared with type II collagen.[35,36] This characteristic of

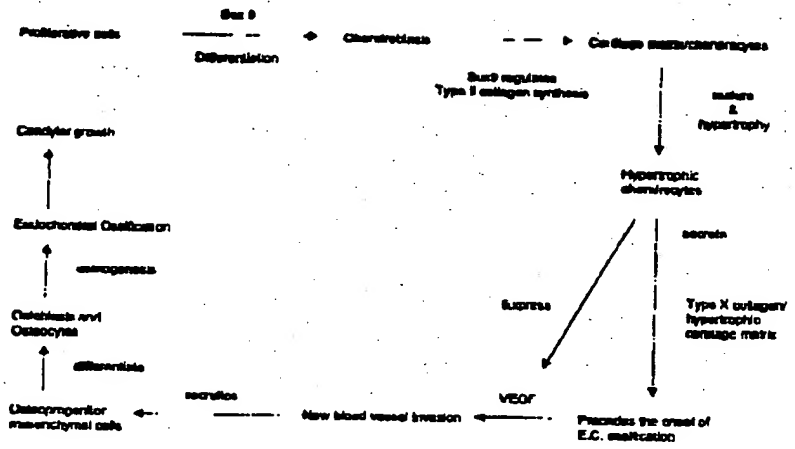

Fig 14. Cascade of condylar growth.

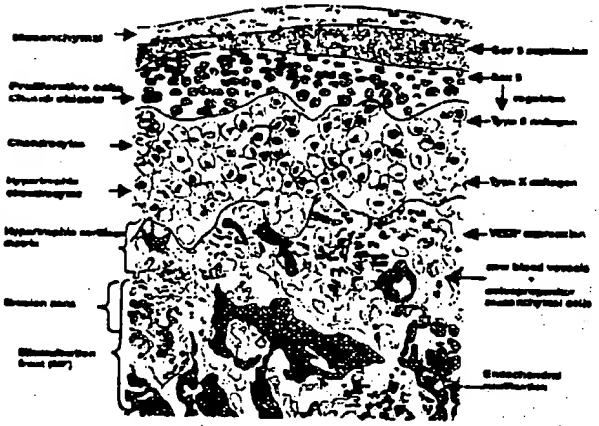

Fig 15. Temporal pattern of cellular differentiation, blood vessel invasion, and bone formation in condyle.

type X collagen makes it the ideal type to form the hypertrophic cartilage matrix in growing mandibular condyles because it probably facilitates the removal of the hypertrophic cartilage and allows for its replacement with bone.

These results correlated the hypertrophic changes that take place in the condylar cartilage to the onset of endochondral ossification but did not address a critical question: What triggers the ossification of the hypertrophied cartilage matrix in the condyles?

Neovascularization of the cartilage of the growth plate plays a fundamental role in endochondral ossification in long bones.[19] Recent reports demonstrated that VEGF is responsible for hypertrophic cartilage neovascularization by targeting invading endothelial cells.[36] The inactivation of VEGF expression suppressed neovascularization and endochondral bone formation in the epiphyseal growth plate in mice. The reintroduction of the VEGF caused capillary invasion and restoration of bone growth.[37] Therefore, in the present study, the localization of VEGF to cells in the upper hypertrophic layer in the condyles suggests a role in the vascularization of the hypertrophic zone (Fig 11). Similar to its localization in long bones, VEGF was secreted by hypertrophic chondrocytes in the rats' condyles (Fig 10). Its temporal pattern of expression was closely related to that of osteogenesis, where the highest levels of VEGF expression coexisted with the highest level of bone formation. The explanation is that VEGF expression causes subsequent neovascularization that is required to trigger the onset of ossification in the hypertrophic condyle cartilage. The invading new blood vessels bring with them nondifferentiated mesenchymal cells in the perivascular sites. These mesenchymal cells later differentiate into osteoblasts that form bone in the growing condyles. Therefore, in the present study, the highest level of VEGF is required earlier in the process of osteogenesis to provide the required pool of osteoprogenitor cells. A detailed examination of the temporal pattern of cellular differentiation, blood vessel invasion, and bone formation in the condyle showed that condylar hypertrophic cartilage is not directly replaced by bone, but by marrow, vascular tissue, and resorptive tissue (Figs 14 and 15). Pechak et al[38] reported that the dimensions of the original cartilage model exactly define the boundaries of the initial marrow cavity. In other words, cartilage does not provide the scaffolding on which bone is formed but, rather, acts as a boundary and a morpho-

logic guide for vasculature and marrow. This is found in the condylar cartilage at the junction between the hypertrophic cartilage and the newly formed bone, a zone referred to in this study as the *mineralization front*. As shown in Figure 15, the resorptive tissue and the vascular tissue lie adjacent to the mineralization front. This is because the invading vascular elements are essential for all bone formation: they contribute to regulation of oxygen tension, nutrient accessibility, and the production of powerful cytokines that affect the osteoblastic lineage directly.[39]

CONCLUSIONS

As shown in Figure 14, the process of condylar growth involves a series of events that can be outlined as follows:

Cells in the proliferative layer express Sox 9 transcription factor required for the differentiation of mesenchymal cells to chondroblasts. Chondroblasts engage in cartilage formation marked by the synthesis of Type II collagen, the main type of collagen that forms the framework of the cartilage matrix in the growing condyle.

Chondrocytes express Sox 9 transcription factor that regulates the synthesis of type II collagen and subsequently affects condylar cartilage formation. These cells continue to mature and progress towards hypertrophy. Hypertrophic chondrocytes secrete type X collagen, the matrix for the hypertrophic cartilage destined for endochondral ossification. Cells in the upper zone of the hypertrophic cartilage secrete VEGF, which regulates the neovascularization of the hypertrophic cartilage and influences the removal of the cartilage matrix.

Erosion of the hypertrophic cartilage continues as neovascularization, and the formation of marrow tissue and resorptive tissue progresses. The invading blood vessels bring osteogenic progenitor mesenchymal cells into the mineralization front and later differentiate into osteoblasts and engage in osteogenesis.

We hope this detailed exposition of the cellular and molecular events participating in bone formation provides the basis for a conceptual model of some key events responsible for the growth of a complex tissue such as the mandibular condyle.

The authors express their gratitude to Dr Laverne Yip for her assistance with the manuscript and Mr Shadow Yeung for preparing the statistics.

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